Applied and Environmental Microbiology, June 1994, p. 1775–1782
0099-2240/94/$04.00+0

Vol. 60, No. 6

Anaerobic Metabolism of Cyclohex-1-Ene-1-Carboxylate, a Proposed Intermediate of Benzoate Degradation, by *Rhodopseudomonas palustris*

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Received 13 December 1993/Accepted 13 March 1994

Anaerobic benzoate degradation by the phototrophic bacterium *Rhodopseudomonas palustris* has been proposed to proceed via aromatic ring reduction reactions leading to cyclohex-1-ene-1-carboxyl-coenzyme A (CoA) formation. The alicyclic product is then proposed to undergo three β-oxidation-like modifications resulting in ring cleavage. Illuminated suspensions of benzoate-grown cells converted [7-^{14}C]cyclohex-1-ene-1-carboxylate to intermediates that comigrated with cyclohex-1-ene-1-carboxyl-CoA, 2-hydroxycyclohexanecarboxyl-CoA, 2-ketocyclohexanecarboxyl-CoA, and pimelyl-CoA by thin-layer chromatography. This set of intermediates was also formed by cells grown anaerobically or aerobically on cyclohex-1-ene-1-carboxylate, indicating that benzoate-grown and cyclohex-1-ene-1-carboxylate-grown cells degrade this alicyclic acid by the same catabolic route. Four enzymatic activities proposed to be required for conversion of cyclohex-1-ene-1-carboxylate to pimelyl-CoA were detected at 3- to 10-fold-higher levels in benzoate-grown cells than in succinate-grown cells. These were cyclohex-1-ene-1-carboxylate-CoA ligase, cyclohex-1-ene-1-carboxyl-CoA hydratase, 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase, and 2-ketocyclohexanecarboxyl-CoA hydrolase (ring cleaving). Pimelyl-CoA was identified in hydrolase reaction mixtures as the product of alicyclic ring cleavage. The results provide a first demonstration of an alicyclic ring cleavage activity.

Rhodopseudomonas palustris grows anaerobically on structurally diverse aromatic compounds, and this phototrophic bacterium, along with two strains of denitrifying pseudomonads, has served as a model organism in studies of anaerobic aromatic compound degradation. The degradation pathways generally include steps to convert aromatic compounds to benzoyl-coenzyme A (CoA), the starting substrate for a central pathway of aromatic ring reduction and cleavage (7, 10, 23). Cyclohex-1-ene-1-carboxylic acid (Δ-1-chca) is a proposed intermediate of anaerobic benzoate degradation by *R. palustris* (6, 12, 16). This alicyclic acid also supports both aerobic and anaerobic growth of this organism (15). Studies of anaerobic benzoate degradation by *R. palustris* (6, 26) and of aerobic cyclohexanecarboxylic acid degradation by an *Alcaligenes* strain (3) have led to the formulation of a proposed pathway for Δ-1-chca degradation which includes CoA thioesterification of the substrate followed by three β-oxidation-like modifications, resulting in cleavage of an alicyclic ring (Fig. 1). Although enzymatic activities required for the generation of the ring cleavage substrate, 2-ketocyclohexanecarboxyl-CoA, were detected in the *Alcaligenes* strain (3), the investigator failed to detect a ring cleavage activity in cell extracts. In a study with *R. palustris*, Hutber and Ribbons (16) also failed to detect alicyclic ring cleavage and reported only very low level constitutive synthesis of enzymatic activities proposed to mediate conversion of cyclohex-1-ene-1-carboxyl-CoA (Δ-1-chca-CoA) to 2-ketocyclohexanecarboxyl-CoA.

Here, we have examined Δ-1-chca degradation by intact cells of *R. palustris*. We have also reexamined enzymatic activities proposed to be involved in the conversion of Δ-1-chca to a ring cleavage product, using refined assay conditions. Work reported here confirms the proposed degradation pathway shown in Fig. 1 and demonstrates a 2-ketocyclohexanecarboxyl-CoA hydrolase (ring cleaving) activity. Profiles of intracellular metabolites formed during short-term incubations of whole cells with ^{14}C-labeled Δ-1-chca indicated that benzoate-grown and Δ-1-chca-grown cells degrade this substrate by the same catabolic route.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. palustris* CGA009 was used in these studies (17). Cultures were grown anaerobically in PM, an inorganic salts medium described previously (17). Carbon sources were supplied at 3 mM final concentration, except for succinate, 10 mM, and acetate, 9 mM. For anaerobic growth, anoxic sodium bicarbonate (10 mM final concentration) was added from sterile stock solutions at the time of inoculation. Anaerobic cultures were illuminated with 40-W incandescent light bulbs and maintained at 30°C. Cells were grown aerobically in PM lacking sodium bicarbonate. Cultures were incubated on a rotary shaker at 250 rpm at 30°C. Growth was monitored by measuring the optical density at 660 nm.

Chemical synthesis of free alicyclic acids. 2-Ketocyclohexanecarboxylic acid was synthesized from ethyl-2-cyclohexanone carboxylate (Aldrich Chemical Co., Inc., Milwaukee, Wis.) as described by Dieckmann (5). The structure of the alicyclic product was confirmed by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS). 2-Hydroxycyclohexanecarboxylic acid was synthesized by reducing ethyl-2-cyclohexanone carboxylate (1 g) with sodium borohydride (40 mg) in 25 ml of 95% ethanol to produce ethyl-2-hydroxycyclohexanecarboxylate. The product was then hydrolyzed with 20 ml of 5% NaOH to produce the corresponding carboxylic acid. The solution was extracted two times with 20

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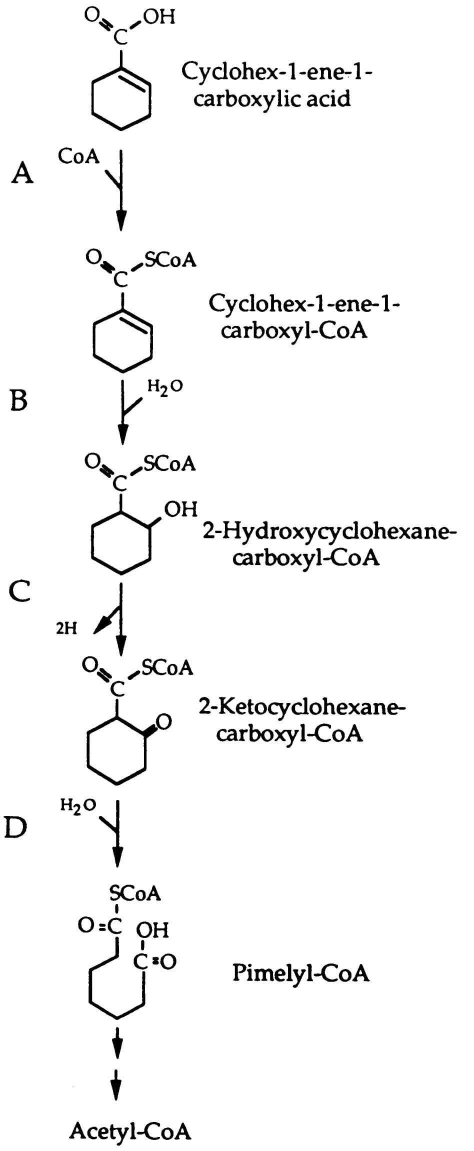

FIG. 1. Proposed pathway for cyclohex-1-ene-1-carboxylate degradation in *R. palustris*. Proposed enzymatic activities are as follows: (A) cyclohex-1-ene-1-carboxylate-CoA ligase; (B) cyclohex-1-ene-1-carboxyl-CoA hydratase; (C) 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase; (D) 2-ketocyclohexanecarboxyl-CoA hydrolase (ring cleaving).

ml of diethyl ether to remove unreacted ethyl-2-hydroxycyclohexanecarboxylate, acidified with concentrated HCl to pH 2 to 3, and extracted two times with 20 ml of diethyl ether. The ether extracts were then combined and rotary evaporated under reduced pressure to yield 0.5 g of the free acid. 2-Hydroxycyclohexanecarboxylic acid was silylated as described below and analyzed by GC-MS to confirm its molecular weight.

Synthesis of CoA thioesters. The CoA thioesters of free alicyclic acids were synthesized as described by Merkel et al. (20), with the exception that the final alkali treatment step was omitted. Crude preparations of CoA thioesters obtained by this procedure were usually stored at −20°C until further purification, using C_{18} reverse-phase cartridges (Sep-Pak; Millipore Corp., Milford, Mass.), with a step gradient of 20 mM KPO_4 buffer (pH 6.0) and methanol. C_{18} cartridges were activated by washing sequentially with 10 ml of methylene chloride, 10 ml of methanol, and 10 ml of H_2O and conditioned with 10 ml of 20 mM KPO_4 buffer (pH 6.0). Cartridges were loaded with 1 ml of a 15- to 20-mg ml^{-1} preparation of the crude acyl-CoA preparation in 20 mM KPO_4 buffer. The loaded cartridges were washed sequentially with 5 ml of 20 mM KPO_4, 15 ml of 20 mM KPO_4–5% methanol, 2 ml of 20 mM KPO_4–10% methanol, 10 ml of 20 mM KPO_4–30% methanol, and 2 ml of 20 mM KPO_4–50% methanol. The 30 and 50% methanol washes were combined, concentrated by rotary evaporation, and lyophilized. The purified acyl-CoA was desalted by loading 1 ml of a 15- to 20-mg ml^{-1} solution (in water) onto an activated C_{18} cartridge and washing with 2 ml of H_2O. It was then eluted with 10 ml of 50% methanol in H_2O. The 50% methanol washes were combined, concentrated by rotary evaporation, and lyophilized.

Uptake assays. Uptake assays were performed as described previously (14, 20). Cells grown anaerobically in completely filled 250-ml bottles were harvested in air by centrifugation, washed once, and resuspended to a final concentration of 0.2 to 0.6 mg of protein ml^{-1} in deaerated 10 mM triethanolamine hydrochloride–10 mM Na_2PO_4 (TEA-PO_4) buffer (pH 7.5) containing 1 mM dithiothreitol (DTT). The resuspended cells were then preincubated in light for 1 h in glass syringes, as described previously (14). Uptake assays were initiated by adding 300 μl of cell suspension to 300 μl of deaerated TEA-PO_4 buffer containing 1 mM DTT and labeled substrate.

Cells to be used in aerobic assays were grown in 1 liter of PM with shaking. Cells were washed as described above, but suspended cells were gently bubbled with air, and assays were carried out in air.

Extraction and analysis of intracellular metabolites. Cells were provided with radiolabeled substrates in short-term incubations as described above with the following changes. A larger volume (600 μl) of cell suspension was added to 600 μl of deaerated TEA-PO_4 buffer containing 1 mM DTT and labeled compound (30 μM [7-^{14}C]Δ-1-chca, 9.54 μM [7-^{14}C]benzoate, or 30 μM [1,7-^{14}C]pimelate). Samples (0.350 to 0.40 ml) were filtered and washed as described previously (14), and the filters were then placed in 1 ml of boiling water for 1 to 2 min before being chilled on ice. Soluble compounds were extracted from cells, concentrated, and chromatographed on cellulose thin-layer plates with fluorescent indicator (Eastman Kodak Co., Rochester, N.Y.), as described previously (20). Radiolabeled compounds were visualized by autoradiography after thin-layer plates were coated with En^{3}Hance spray (Biotechnology Systems, NEN Research Products, Boston, Mass.) and exposed to X-ray film for 2 to 4 weeks at −70°C. Unlabeled standards were detected by UV absorbance. Lyophilized products were also dissolved in water and analyzed by high-performance liquid chromatography (HPLC), as described below.

Preparation of cell extracts. Cells were harvested by centrifugation, washed once in 10 mM Tris buffer (pH 7.0), and suspended in 10 mM Tris buffer (pH 7.0) containing 1 mM DTT in a volume which would concentrate the cells 250 times. Cells were broken by sonication, and cell debris was removed by centrifugation at 15,600 × *g* for 10 min at 4°C. The supernatant was centrifuged at 103,000 × *g* for 1 h at 4°C to pellet the cell membranes. The resulting supernatant, termed crude cell extract, was used in enzyme assays. For cyclohex-1-ene-1-carboxylate-CoA ligase assays, 20 mM TEA buffer (pH 7.0) was substituted for 10 mM Tris buffer (pH 7.0).

Enzyme assays. Cyclohex-1-ene-1-carboxylate-CoA ligase activity was measured by the isotopic assay procedure for

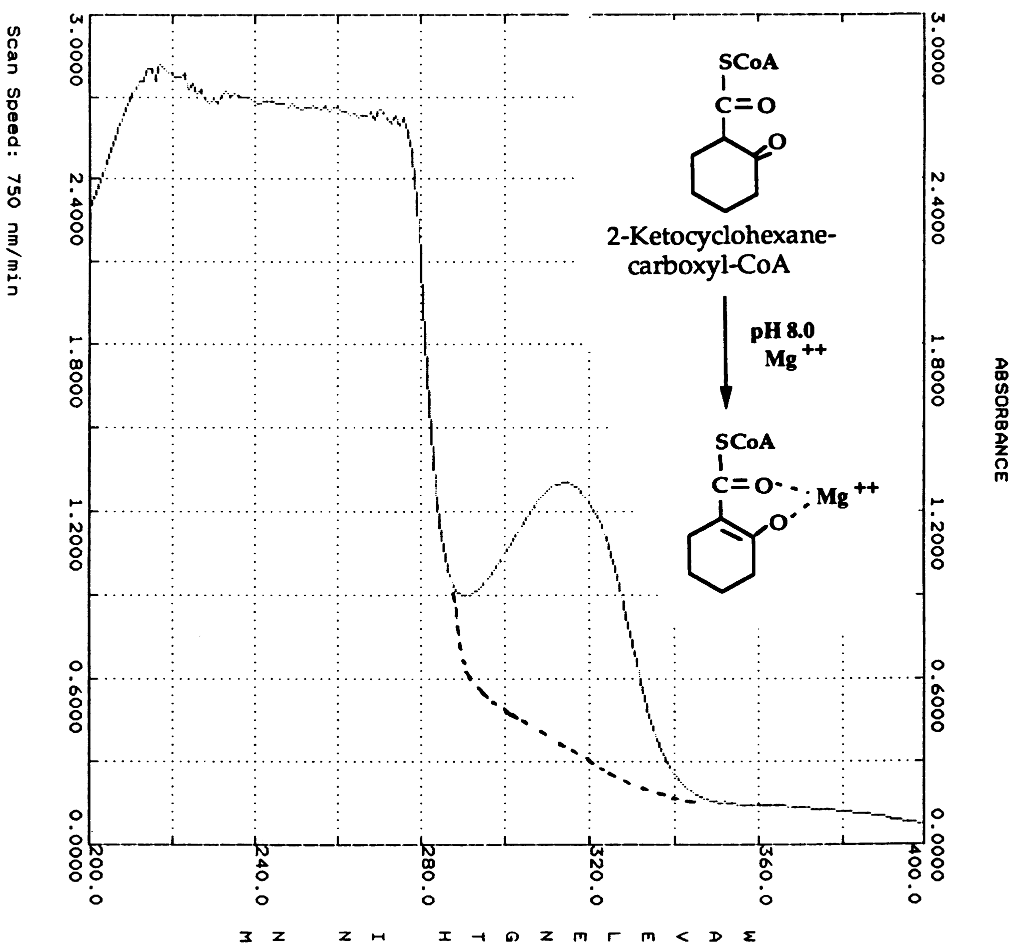

FIG. 2. Formation of an Mg^{2+}-enolate complex of 2-ketocyclohexanecarboxyl-CoA. ——, absorbance spectrum of 2-ketocyclohexanecarboxyl-CoA–magnesium complex at pH 8.0 (maximum absorbance was at 314 nm). – – –, absorbance spectra of 2-ketocyclohexanecarboxyl-CoA at pH 8.0 and of the alicyclic ring cleavage reaction mixture at the completion of the assay.

benzoate-CoA ligase described by Geissler et al. (8), except that 56.2 μM [7-^{14}C]Δ-1-chca was substituted for benzoate. This assay is based on enzymatic conversion of [7-^{14}C]Δ-1-chca to a product (Δ-1-chca-CoA) that remained hydrophilic at acid pH. Combined Δ-1-chca-CoA hydratase and 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase activities were assayed in the forward direction, using Δ-1-chca-CoA as the substrate (3). The reaction mixture contained 100 mM Tris buffer (pH 8.0), 2.8 mM NAD, 2 mM DTT, and 1.14 mM Δ-1-chca-CoA. The reaction was initiated by the addition of 5 to 10 μl of cell extract. The rate of increase in A_{340} was recorded with a Beckman DU-64 spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.).

2-Hydroxycyclohexanecarboxyl-CoA dehydrogenase activity was assayed in the forward and reverse directions. In the forward direction, the reaction mixture contained 100 mM Tris buffer (pH 8.0), 2.8 mM NAD, 2 mM DTT, and 1.12 mM 2-hydroxycyclohexanecarboxyl-CoA. In the reverse direction, the reaction mixture contained 100 mM Tris buffer (pH 7.0), 0.28 mM NADH, 2 mM DTT, and 1.12 mM 2-ketocyclohexanecarboxyl-CoA (3). Reactions were initiated by the addition of crude cell extract, and the change in absorbance was recorded at 340 nm. Acetoacetyl-CoA dehydrogenase activity (1.17 mM substrate concentration) was also assayed using the conditions for the reverse assay.

2-Ketocyclohexanecarboxyl-CoA gives an absorbance peak at 314 nm when incubated in the presence of Mg^{2+} at pH 8 (Fig. 2). This is probably due to the formation of an Mg^{2+}-enolate complex similar to that formed by acetoacetyl-CoA in the presence of Mg^{2+} ions at pH 8 (19, 24, 25). The characteristic absorbance maximum allowed us to monitor the enzymatic cleavage of 2-ketocyclohexanecarboxyl-CoA in crude cell extracts. 2-Ketocyclohexanecarboxyl-CoA hydrolase (alicyclic ring cleavage) activity was measured as the decrease in A_{314}. The reaction mixture contained 100 mM Tris buffer (pH 8.0), 100 mM $MgCl_2$, and 1.12 mM 2-ketocyclohexanecarboxyl-CoA. Activity was calculated by using an extinction coefficient of 1,210 M^{-1} cm^{-1} for 2-ketocyclohexanecarboxyl-CoA. Acetoacetyl-CoA thiolase activity (0.12 mM substrate concentration) was measured under similar conditions, except that 2 mM CoA was also included in the reaction mixture, and the absorbance decrease was monitored at 305 nm. An extinction coefficient of 16,900 M^{-1} cm^{-1} was used for acetoacetyl-CoA (2).

Identification of the ring cleavage product. The ring cleavage reaction (5 ml), carried out as described above, was stopped after 10 min by adjusting the pH to 2 to 3 with 1 N perchloric acid. The acidified mixture was centrifuged for 10 min at 16,800 × *g* at 4°C to pellet the protein. The supernatant was extracted two times with 5 ml of diethyl ether. This ether extract was termed acid extract. The aqueous phase was then adjusted to a pH of 12 to 14 with 1 M KOH. The solution was centrifuged, and the supernatant was incubated at 70°C for 10 min to hydrolyze CoA esters. The supernatant was adjusted to pH 2 to 3 with 1 N perchloric acid and extracted two times with an equal volume of diethyl ether. This extract was termed base-hydrolyzed extract. The diethyl ether in the acid and base-hydrolyzed extracts was removed by drying under a stream of nitrogen. The dry residue remaining was dissolved in 900 μl of dimethyl formamide and silylated with 100 μl of *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Pierce, Rockford, Ill.). The silyl derivative was analyzed by GC-MS.

Analytical procedures. Synthesized compounds and radiolabeled cell extracts were analyzed by HPLC with a binary gradient model 114M HPLC with an Ultrasphere ODS-C_{18} reversed-phase column (Beckman, San Ramon, Calif.), using 20 mM KPO_4 buffer (pH 6.0) and methanol as solvents A and B, respectively. A linear gradient of 15 to 50% solvent B in 30 min was followed by a linear gradient of 50 to 80% solvent B in 5 min. The flow rate was 0.5 ml min^{-1}. Absorbance of the effluent was monitored at 254 nm. Samples were prepared for HPLC analysis by suspension in deionized water and centrifugation in a microcentrifuge for 5 min to remove particulate material.

The UV spectrum of 2-ketocyclohexanecarboxyl-CoA was scanned from 200 to 400 nm with a Beckman model DU-7 spectrophotometer.

GC-MS was performed at the University of Iowa High Resolution Mass Spectrometry Facility on a Fisons TRIO-1 GC-MS equipped with a 15-m methyl silicone DB-1 column (J. and W. Scientific, Folsom, Calif.). Helium was the carrier gas at a flow rate of 1 ml min^{-1}. The column was programmed from 70 to 250°C at 20°C min^{-1}, and the temperature of the injection port and transfer line was 250°C. Low-resolution electron impact ionization was performed at an ionization energy of 70 eV. Samples (1 μl) were injected into a splitless injection port.

NMR analysis was performed at the University of Iowa High-Field NMR Facility on a Bruker WM-360 spectrometer. Proton (^1H) and carbon (^{13}C) NMR spectra were recorded at 360 and 90 MHz, respectively.

Protein from cell extracts was determined with a dye-binding assay (4), using reagents from Bio-Rad Laboratories (Richmond, Calif.). Whole-cell protein was assayed as described previously (20).

Chemicals. Some growth substrates and reagents for thioester synthesis were obtained from commercial sources (Aldrich Chemical Co.; Sigma Chemical Co., St. Louis, Mo.). Acetoacetyl-CoA and benzoyl-CoA were purchased from Sigma. [7-^{14}C]benzoic acid (specific activity, 21.8 mCi $mmol^{-1}$) was from New England Nuclear Research Products (Wilmington, Del.). Δ-1-chca was purchased from Frinton Laboratories (Vineland, N.J.). [7-^{14}C]Δ-1-chca (specific activity, 55 mCi $mmol^{-1}$) and [1,7-^{14}C]pimelic acid (specific activity, 55 mCi $mmol^{-1}$) were from American Radiolabeled Chemicals Inc. (St. Louis, Mo.). Pimelyl-CoA was synthesized according to the method of Ploux and Marquet (22).

RESULTS

R. palustris grew aerobically and anaerobically on 2-hydroxycyclohexanecarboxylate and pimelate, the free acid forms of two of the proposed intermediates of Δ-1-chca degradation (Table 1). The aerobic and anaerobic doubling times were comparable to those obtained with succinate. The inability of *R. palustris* to readily metabolize 2-ketocyclohexanecarboxylate under either incubation condition may be due to the lack of a CoA ligase which would allow the conversion of 2-ketocyclohexanecarboxylate to the corresponding CoA ester.

TABLE 1. Growth rates of *R. palustris* wild-type cells

Growth substrate	Growth rate (h)[a]	
	Anaerobic	Aerobic
Benzoate	16.0 ± 2	No growth
Δ-1-Chca	8.3 ± 1	29.2 ± 10
2-Hydroxycyclohexanecarboxylate	9.3 ± 3	25.7 ± 7
2-Ketocyclohexanecarboxylate	>200	>200
Pimelate	7.0 ± 1	21.1 ± 5
Succinate	7.0 ± 1	19.5 ± 9

[a] Doubling times are averages of at least three determinations ± standard deviations.

Soluble intracellular intermediates formed from Δ-1-chca. To characterize the initial steps of Δ-1-chca metabolism, we extracted and analyzed by thin-layer chromatography the small-molecule pools of cells that had taken up [7-^{14}C]Δ-1-chca for periods of 1 to 3 min (Fig. 3). Measurements of R_f values suggested that cells metabolized Δ-1-chca to the same set of intermediates, regardless of whether they had been grown aerobically or anaerobically. This indicates that the same Δ-1 chca degradation pathway may be used under

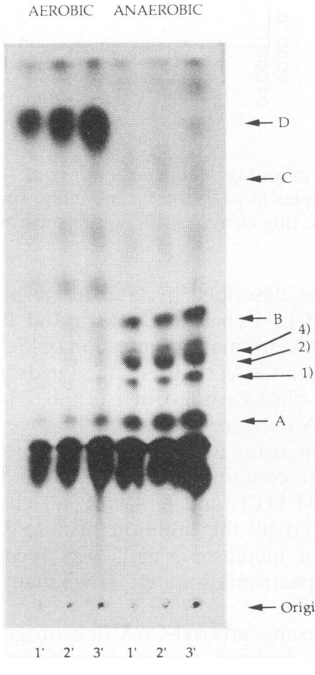

FIG. 3. Autoradiogram comparing the intracellular intermediates formed by *R. palustris* cells given [7-^{14}C]cyclohex-1-ene-1-carboxylate after aerobic or anaerobic growth on cyclohex-1-ene-1-carboxylate. Uptake assays were performed either aerobically or anaerobically according to the growth conditions. Compounds 1, 2, and 4 comigrate with authentic standards 2-ketocyclohexanecarboxyl-CoA (2-keto-CoA)–2-hydroxycyclohexanecarboxyl-CoA (2-OH-CoA), pimelyl-CoA (Pim-CoA), and Δ-1-chca-CoA (Δ-1-CoA). Compounds A, B, C, and D were not identified. 2-Ketocyclohexanecarboxyl-CoA and 2-hydroxycyclohexanecarboxyl-CoA were not resolved by the thin-layer chromatography solvent system used.

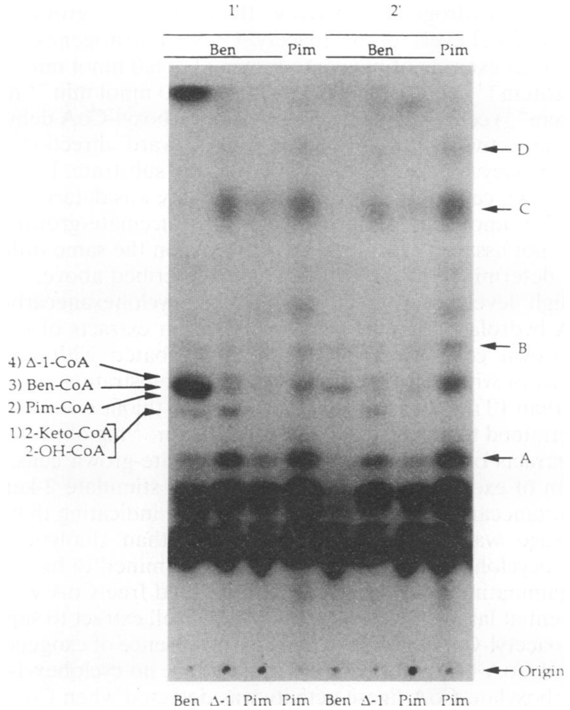

FIG. 4. Autoradiogram comparing the intracellular intermediates formed by *R. palustris* cells given [7-^{14}C]benzoate, [7-^{14}C]cyclohex-1-ene-1-carboxylate, or [1,7-^{14}C]pimelate after anaerobic growth on benzoate or pimelate. Compounds 1, 2, 3, and 4 comigrate with authentic standards 2-ketocyclohexanecarboxyl-CoA (2-keto-CoA)–2-hydroxycyclohexanecarboxyl-CoA (2-OH-CoA), pimelyl-CoA (Pim-CoA), benzoyl-CoA (Ben-CoA), and Δ-1-chca-CoA (Δ-1-CoA). Compounds A, B, C, and D were not identified. 2-Ketocyclohexanecarboxyl-CoA and 2-hydroxycyclohexanecarboxyl-CoA were not resolved by the thin-layer chromatography solvent system used.

TABLE 2. Rates of anaerobic benzoate, Δ-1-chca, and pimelate uptake by *R. palustris* wild-type cells[a]

Labeled substrate[b]	Uptake (nmol min^{-1} mg of protein^{-1})				
	Benzoate	Δ-1-Chca	Pimelate	Succinate	Acetate
Benzoate	2.79	1.18	0.28	0.63	ND[c]
Δ-1-Chca	56.9	66.1	1.00	3.58	ND
Pimelate	30.2	60.3	80.2	77.3	10.4

[a] Cells were grown under anaerobic conditions on the indicated carbon source. Assays were carried out anaerobically in light for 2 min. Uptake rates are reported as nanomoles of compound accumulated in cells. Activities are averages of at least three assays of three independently prepared cell suspensions.

[b] Substrates were supplied at the following concentrations: 9.54 μM benzoate, 53.6 μM Δ-1-chca, and 50.9 μM pimelate. Benzoate and Δ-1-chca were supplied at concentrations that were determined to be saturating for benzoate-grown cells. Pimelate was supplied at a concentration determined to be saturating for both pimelate-grown and benzoate-grown cells.

[c] ND, not determined.

aerobic and anaerobic conditions. However, the rate-limiting steps appear to differ depending on the availability of oxygen, since the concentrations of individual products were different as indicated by the relative intensities of individual spots (Fig. 3). In these experiments, radiolabeled compounds which comigrated with Δ-1-chca-CoA, 2-ketocyclohexanecarboxyl-CoA, 2-hydroxycylohexanecarboxyl-CoA, and pimelyl-CoA, four of the proposed intermediates of Δ-1-chca degradation (Fig. 1), were identified. The labeled intermediates near the solvent front are probably free acids since labeled free acids used as substrates migrated in this position.

A similar product profile was seen in benzoate-grown cells provided with labeled Δ-1-chca (Fig. 4). The R_fs of the labeled intermediates formed from Δ-1-chca by anaerobic benzoate-grown cells matched those of the labeled intermediates formed by cells grown anaerobically on Δ-1-chca. Radiolabeled Δ-1-chca-CoA was also identified by HPLC in intracellular extracts of benzoate-grown cells. These results indicate that benzoate- and Δ-1-chca-grown cells degrade Δ-1-chca via the same catabolic route.

Labeled intermediates present in extracts of cells that were given [7-^{14}C]Δ-1-chca were compared with those present in extracts of cells that had taken up labeled [1,7-^{14}C]pimelate, a proposed intermediate in the degradation of Δ-1-chca (Fig. 4). The autoradiogram shown in Fig. 4 indicates that there are just two spots unique to Δ-1-chca degradation. One of these comigrates with Δ-1-chca-CoA, and the other comigrates with 2-hydroxycyclohexanecarboxyl-CoA and 2-ketocyclohexanecarboxyl-CoA. In both extracts, a labeled intermediate formed which comigrated with the authentic standard pimelyl-CoA. These results suggest that pimelyl-CoA is an intermediate in the proposed β-oxidation-like reactions required for Δ-1-chca degradation and that benzoate-grown cells metabolize pimelate by the same route as pimelate-grown cells.

Uptake rates. Rates of Δ-1-chca, benzoate, and pimelate uptake by *R. palustris* cells grown anaerobically in light on various carbon sources were measured (Table 2). Growth on benzoate and Δ-1-chca induced uptake of all three radiolabeled substrates. The rate of labeled Δ-1-chca uptake by Δ-1-chca-grown cells was similar to the rate of uptake measured for benzoate-grown cells. However, benzoate-grown cells accumulated labeled pimelate at a rate half that of Δ-1-chca-grown cells. Acetate-grown cells accumulated pimelate at a substantially lower rate than cells grown on benzoate, Δ-1-chca, pimelate, or succinate, suggesting that pimelate metabolism could be repressible by acetate.

Comparisons of rates of Δ-1-chca uptake by illuminated suspensions of succinate- and benzoate-grown cells indicate that Δ-1-chca uptake is induced by growth on benzoate (Fig. 5). Uptake of Δ-1-chca is also an energy-dependent process since cells incubated in the dark and assayed anaerobically under nitrogen in foil-wrapped tubes took up labeled Δ-1-chca at very low rates (Fig. 5). The apparent K_m of Δ-1-chca uptake by anaerobic benzoate-grown cells was calculated to be 8 μM.

R. palustris cells grown aerobically on 3 mM Δ-1-chca were also assayed for the uptake of labeled Δ-1-chca. Cell suspensions supplemented with 67 μM acetate took up Δ-1-chca at a rate of 39.9 nmol min^{-1} mg of protein^{-1}, whereas cells lacking any additional energy source took up Δ-1-chca at a rate of 14.7 nmol min^{-1} mg of protein^{-1}.

Enzymatic activities in cell extracts. A Δ-1-chca-CoA ligase activity was measured in the soluble protein fraction of cells grown anaerobically on benzoate. Succinate-grown cells had lower, but detectable, ligase activity (Table 3). Anaerobic Δ-1-chca-grown cells had levels of Δ-1-chca-CoA ligase activity similar to those measured in benzoate-grown cells (data not shown). No activity was detected when CoA was omitted from the reaction mixture. Activities were very low (0.20 nmol min^{-1} mg of protein^{-1}) when ATP was omitted from the reaction mixture.

Δ-1-chca-CoA hydratase and 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase activities were measured in a coupled assay with Δ-1-chca-CoA as the substrate. Crude extracts of benzoate-grown *R. palustris* expressed fivefold-higher activity

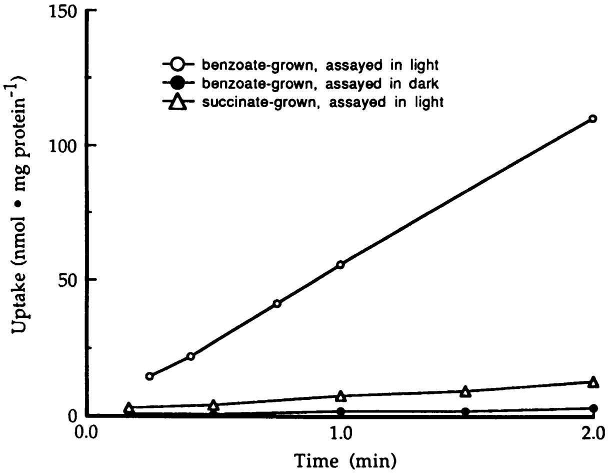

FIG. 5. Anaerobic cyclohex-1-ene-1-carboxylate uptake by *R. palustris* cells grown under anaerobic conditions. Benzoate- and succinate-grown cell suspensions were preincubated anaerobically in the light, and uptake assays were performed anaerobically in the light. Benzoate-grown cell suspensions were preincubated anaerobically in the dark for 1 h, with the assays performed anaerobically in foil-wrapped tubes.

for the coupled enzymatic reaction than extracts from succinate-grown cells (Table 3). No activity was detected when benzoate-grown cell extracts were given Δ-1-chca-CoA as the substrate and NADH as a cofactor. This shows that Δ-1-chca-CoA was not consumed by an alternate enzymatic reaction that used the NADH produced by 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase activity.

2-Hydroxycyclohexanecarboxyl-CoA dehydrogenase was assayed in the reverse direction with 2-ketocyclohexanecarboxyl-CoA as the substrate. Benzoate-grown cells had a 10-fold-higher dehydrogenase activity than succinate-grown cells (Table 3). Levels of acetoacetyl-CoA dehydrogenase were similar in extracts of benzoate-grown (1,256.0 nmol min^{-1} mg of protein^{-1}) and succinate-grown (1,151.0 nmol min^{-1} mg of protein^{-1}) cells. 2-Hydroxycyclohexanecarboxyl-CoA dehydrogenase activity was assayed in the forward direction with 2-hydroxycyclohexanecarboxyl-CoA as the substrate. In benzoate-grown cells, this dehydrogenase activity was determined to be 22.0 nmol min^{-1} mg of protein^{-1}. Succinate-grown cells were not assayed. This level of activity is on the same order as that determined in the coupled assay described above.

TABLE 3. Specific activities of enzymes involved in cyclohex-1-ene-1-carboxylate degradation in wild-type *R. palustris* cells[a]

Enzyme	Sp act (nmol min^{-1} mg of protein^{-1})	
	Benzoate-grown cells	Succinate-grown cells
Cyclohex-1-ene-1-carboxylate-CoA ligase[b]	5.7	2.1
Δ-1-Chca-CoA hydratase and 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase[c]	29.9	6.4
2-Hydroxycyclohexanecarboxyl-CoA dehydrogenase[d]	277.6	28.4
2-Ketocyclohexanecarboxyl-CoA hydrolase (ring cleaving)[e]	1,019.0	108.1

[a] Nanomoles of substrate used or product formed. Activities reported are averages of at least two assays of three independently prepared extracts. Cells were grown under anaerobic conditions on the indicated carbon source.

[b] Measured as the amount of radiolabeled Δ-1-chca-CoA remaining in the aqueous phase after removal of unreacted Δ-1-chca by ethyl acetate extraction.

[c] Assayed in the forward direction, using Δ-1-chca-CoA as the substrate. Activity was calculated from the nanomoles of NADH formed.

[d] Assayed in the reverse direction, using 2-ketocyclohexanecarboxyl-CoA as the substrate. Activity was calculated from the nanomoles of NADH that disappeared.

[e] Measured as the decrease in A_{314} due to the disappearance of a 2-ketocyclohexanecarboxyl-CoA–magnesium ion complex. Assays were performed at pH 8.0.

High levels of ring cleavage (2-ketocyclohexanecarboxyl-CoA hydrolase) activity were measured in extracts of benzoate-grown cells. Reaction mixtures incubated with no cell extract or with boiled cell extract cleaved substrate at a rate of less than 10 nmol min^{-1}. Acetoacetyl-CoA thiolase activity was determined to be 880.0 and 708.4 nmol min^{-1} mg of protein^{-1} in extracts of benzoate-grown and succinate-grown cells. Addition of exogenous CoA (2 mM) did not stimulate 2-ketocyclohexanecarboxyl-CoA hydrolase activity, indicating that ring cleavage was indeed hydrolytic rather than thiolytic. The 2-ketocyclohexanecarboxyl-CoA was determined to be free of contaminating CoA by HPLC analysis, and free CoA was not present at large enough quantities in the cell extract to support acetoacetyl-CoA thiolase activity in the absence of exogenously added CoA (data not shown). In addition, no cyclohex-1-ene-1-carboxylate-CoA ligase activity was detected when CoA was omitted from the reaction mixture.

Pimelyl-CoA is the probable ring cleavage product. The product of the alicyclic ring cleavage reaction was identified in ether extracts of alkali-treated 2-ketocyclohexanecarboxyl-CoA hydrolase reaction mixtures as pimelic acid (Fig. 6). Twice as much pimelic acid was detected in ether extracts of base-hydrolyzed reaction mixtures as in ether extracts of acid-treated reaction mixtures. Since CoA thioesters are alkali labile, pimelyl-CoA is the probable actual cleavage product. No pimelic acid was detected in ether extracts of reaction mixtures when 2-ketocyclohexanecarboxylate was the substrate, indicating that the CoA moiety is an important feature of substrate recognition.

DISCUSSION

The results of this study indicate that *R. palustris* metabolizes cyclohex-1-ene-1-carboxylate through three β-oxidation-like modifications, resulting in ring cleavage to form pimelyl-CoA (Fig. 1). Cyclohex-1-ene-1-carboxylate-CoA ligase, Δ-1-chca-CoA hydratase, 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase, and 2-ketocyclohexanecarboxyl-CoA hydrolase (ring cleaving) activities were detected in extracts of *R. palustris* cells grown anaerobically in light on benzoate. Benzoate-grown cells possessed 3- to 10-fold-higher activities than succinate-grown cells. These results are in contrast to the results of Hutber and Ribbons (16), who failed to detect alicyclic ring cleavage and reported low-level constitutive synthesis of enzymatic activities proposed to mediate the conversion of Δ-1-chca-CoA to 2-ketocyclohexanecarboxyl-CoA.

This is the first report to describe a 2-ketocyclohexanecarboxyl-CoA hydrolase activity. The probable ring cleavage product is pimelyl-CoA, since twice as much pimelic acid was extracted from base-hydrolyzed reaction mixtures, compared with those treated with acid, as would be expected of product esterified with CoA, a modification known to be alkali labile. Free pimelic acid extracted from acid-treated reaction mixtures could have been released by a CoA thioesterase activity

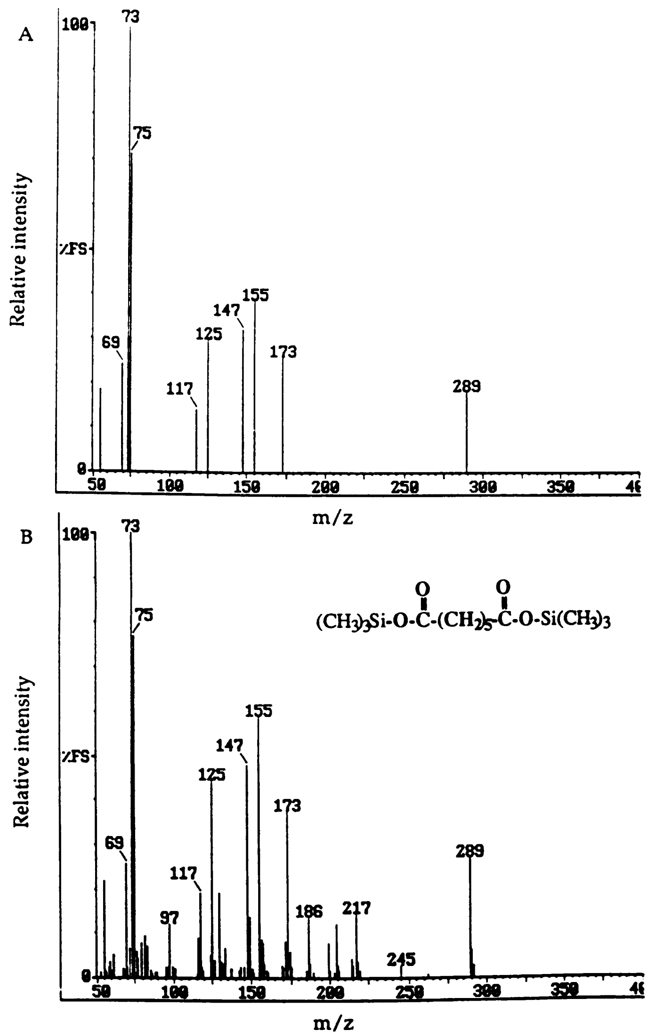

FIG. 6. Mass spectra of pimelate. (A) Disilyl-pimelate from a cell extract catalyzing the cleavage of 2-ketocyclohexanecarboxyl-CoA; (B) disilyl-pimelate standard.

present in the crude cell extracts. Such an activity would also explain the appearance of labeled free acids in whole cells given Δ-1-chca (Fig. 3 and 4). Whittle et al. (26) reported the production of pimelic acid from extracts of *R. palustris* cells given Δ-1-chca, CoA, ATP, NAD, and $MgSO_4$, but these investigators did not specifically look for CoA thioesters. Definitive identification of the ring cleavage product will require purification of the 2-ketocyclohexanecarboxyl-CoA hydrolase. This would allow for the direct identification of the actual CoA thioester produced by the enzymatic reaction.

Benzoate-grown *R. palustris* given labeled Δ-1-chca formed labeled intermediates which comigrated with Δ-1-chca-CoA, 2-hydroxycyclohexanecarboxyl-CoA, 2-ketocyclohexanecarboxyl-CoA, and pimelyl-CoA. These compounds are also proposed intermediates of anaerobic benzoate degradation (6, 12, 16). Recently, working with anaerobic cell extracts provided with benzoate, CoA, and titanium(III) citrate as the reductant, Fuchs and coworkers have used two-dimensional NMR to directly identify 6-hydroxycyclohex-1-ene-1-carboxyl-CoA as an additional alicyclic intermediate of anaerobic benzoate degradation (18). From a time course assay which followed intermediate production and consumption, these workers proposed an alternative route for benzoate degradation which included alicyclic intermediates hydroxylated at the C-6 and C-2 positions. Firm delineation of the sequence of intermediates formed in vivo during growth on benzoate will require detailed biochemical and genetic analyses. It is possible, however, that the enzyme activities reported here can catalyze both sets of proposed reactions. Our attempts to visualize radiolabeled intermediates formed from ^{14}C-benzoate by whole cells were disappointing. As has been noted previously, benzoyl-CoA accumulates to a very high internal concentration (11). This suggests that subsequent ring reduction steps are rate limiting and may explain why very low amounts of additional intermediates were seen.

Uptake of Δ-1-chca by *R. palustris* is an energy-dependent process that is inducible by growth on benzoate. The low K_m of cells for Δ-1-chca (8 μM) is also indicative of a high-affinity uptake mechanism. We identified Δ-1-chca-CoA by thin-layer chromatography and HPLC in extracts of benzoate-grown cells given labeled Δ-1-chca and demonstrated that crude extracts from benzoate-grown cells also possess Δ-1-chca-CoA ligase activity. These data suggest that Δ-1-chca uptake and thioesterification may be linked and are consistent with an uptake mechanism that involves entry of Δ-1-chca into cells by simple diffusion followed by a rapid "trapping" of the compound as its CoA derivative. Such a mechanism has been proposed for aromatic acid uptake by *R. palustris*, in which a linkage between benzoate or 4-hydroxybenzoate uptake and CoA ester formation by benzoate-CoA or 4-hydroxybenzoate-CoA ligases has been shown (8, 20).

Activities of Δ-1-chca-CoA ligase were low relative to the activities of the other enzymes in the alicyclic β-oxidation sequence (Table 3). Low aromatic acid CoA ligase activities are commonly observed in crude cell extracts from *R. palustris* and denitrifying pseudomonads (1, 9, 27) and might be explained in part by rapid hydrolysis of CoA esters by a thioesterase activity present in extracts. It has also been suggested that compounds liberated during cell breakage may inhibit CoA ligase activities, because dramatic increases in activity have been seen after dialysis or partial enzyme purification (1, 9). However, in the present study, overnight dialysis of crude cell extracts did not result in an increase in Δ-1-chca-CoA ligase activity.

From his work with an *Alcaligenes* strain, Blakley (3) proposed that pimelyl-CoA formed from the alicyclic ring cleavage is converted by β-oxidation reactions to 3-ketopimelyl-CoA. This compound is then cleaved to glutaryl-CoA and acetyl-CoA. Conversion of glutaryl-CoA to glutaconyl-CoA, followed by a decarboxylation to produce crotonyl-CoA, was then proposed. Support for this sequence comes from the work of Härtel et al. (13), who demonstrated significant levels of glutaryl-CoA dehydrogenase activity in cell extracts of *Pseudomonas* sp. strains KB 740 and K 172 and *R. palustris* after anaerobic growth on benzoate. Crotonyl-CoA is probably converted to β-hydroxybutyryl-CoA and then to acetoacetyl-CoA, which is cleaved to two molecules of acetyl-CoA (21). A comparison of intracellular metabolites formed by *R. palustris* from radiolabeled Δ-1-chca and pimelate shows that the two substrates are degraded to form a large number of common compounds. Most likely these are the metabolites, mentioned above, which are proposed to be formed during the conversion of pimelyl-CoA to acetyl-CoA.

ACKNOWLEDGMENTS

This work was supported by grant DAAL03-92-G-0312 from the U.S. Army Research Office, with cofunding from the Department of Energy, Division of Energy Biosciences.

We are grateful to K. J. Gibson for the preparation of pimelyl-CoA. We thank K. J. Gibson and J. Gibson for helpful discussions.

REFERENCES

1. **Altenschmidt, U., B. Oswald, and G. Fuchs.** 1991. Purification and characterization of benzoate-coenzyme A ligase and 2-aminobenzoate-coenzyme A ligases from a denitrifying *Pseudomonas* sp. J. Bacteriol. **173:**5494–5501.
2. **Binstock, J. F., and H. Schulz.** 1981. Fatty acid oxidation complex from *Escherichia coli*. Methods Enzymol. **71:**403–411.
3. **Blakley, E. R.** 1978. The microbial degradation of cyclohexanecarboxylic acid by a β-oxidation pathway with simultaneous induction to the utilization of benzoate. Can. J. Microbiol. **24:**847–855.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Dieckmann, W.** 1901. Ueber cyklische β-Ketocarbonsäuereester. Liebigs Ann. Chem. **317:**98–109.
6. **Dutton, P. L., and W. C. Evans.** 1969. The metabolism of aromatic compounds by *Rhodopseudomonas palustris*. A new, reductive, method of aromatic ring metabolism. Biochem. J. **113:**525–536.
7. **Fuchs, G., M. Mohamed, U. Altenschmidt, J. Koch, A. Lack, R. Brackmann, C. Lochmeyer, and B. Oswald.** 1994. Biochemistry of anaerobic biodegradation of aromatic compounds, p. 513–553. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
8. **Geissler, J. F., C. S. Harwood, and J. Gibson.** 1988. Purification and properties of benzoate-coenzyme A ligase, a *Rhodopseudomonas palustris* enzyme involved in the anaerobic degradation of benzoate. J. Bacteriol. **170:**1709–1714.
9. **Gibson, J., M. Dispensa, G. C. Fogg, D. T. Evans, and C. S. Harwood.** 1994. 4-Hydroxybenzoate-coenzyme A ligase from *Rhodopseudomonas palustris*: purification, gene sequence, and role in anaerobic degradation. J. Bacteriol. **176:**634–641.
10. **Gibson, J., and C. S. Harwood.** 1994. Anaerobic utilization of aromatic carboxylates by bacteria, p. 298–313. *In* R. G. Chaudhry (ed.), Biological degradation and bioremediation of toxic chemicals. Dioscorides Press, Portland, Oreg.
11. **Gibson, K. J., and J. Gibson.** 1992. Potential early intermediates in anaerobic benzoate degradation by *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. **58:**696–698.
12. **Guyer, M., and G. Hegeman.** 1969. Evidence for a reductive pathway for the anaerobic metabolism of benzoate. J. Bacteriol. **99:**906–907.
13. **Härtel, U., E. Eckel, J. Koch, G. Fuchs, D. Linder, and W. Buckel.** 1993. Purification of glutaryl-CoA dehydrogenase from *Pseudomonas* sp., an enzyme involved in the anaerobic degradation of benzoate. Arch. Microbiol. **159:**174–181.
14. **Harwood, C. S., and J. Gibson.** 1986. Uptake of benzoate by *Rhodopseudomonas palustris* grown anaerobically in light. J. Bacteriol. **165:**504–509.
15. **Harwood, C. S., and J. Gibson.** 1988. Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. **54:**712–717.
16. **Hutber, G. N., and D. W. Ribbons.** 1983. Involvement of coenzyme A esters in the metabolism of benzoate and cyclohexanecarboxylate by *Rhodopseudomonas palustris*. J. Gen. Microbiol. **129:**2413–2420.
17. **Kim, M.-K., and C. S. Harwood.** 1991. Regulation of benzoate-CoA ligase in *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. **83:**199–204.
18. **Koch, J., W. Eisenreich, A. Bacher, and G. Fuchs.** 1993. Products of enzymatic reduction of benzoyl-CoA, a key reaction in anaerobic aromatic metabolism. Eur. J. Biochem. **211:**649–661.
19. **Lynen, F., and S. Ochoa.** 1953. Enzymes of fatty acid metabolism. Biochim. Biophys. Acta **12:**299–314.
20. **Merkel, S. M., A. E. Eberhard, J. Gibson, and C. S. Harwood.** 1989. Involvement of coenzyme A thioesters in anaerobic metabolism of 4-hydroxybenzoate by *Rhodopseudomonas palustris*. J. Bacteriol. **171:**1–7.
21. **Nunn, W. D.** 1987. Two-carbon compounds and fatty acids as carbon sources, p. 285–301. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
22. **Ploux, O., and A. Marquet.** 1992. The 8-amino-7-oxopelargonate synthase from *Bacillus sphaericus*. Purification and preliminary characterization of the cloned enzyme overproduced in *Escherichia coli*. Biochem. J. **283:**327–331.
23. **Schink, B., A. Brune, and S. Schnell.** 1992. Anaerobic degradation of aromatic compounds, p. 220–242. *In* G. Winkelmann (ed.), Microbial degradation of natural products. VCH Weinheim, New York.
24. **Stern, J. R.** 1955. Enzymes of acetoacetate formation and breakdown. Methods Enzymol. **1:**573–585.
25. **Stern, J. R., M. J. Coon, and A. Del Campillo.** 1953. Acetoacetyl coenzyme A as intermediate in the enzymatic breakdown and synthesis of acetoacetate. J. Am. Chem. Soc. **75:**1517–1518.
26. **Whittle, P. J., D. O. Lunt, and W. C. Evans.** 1976. Anaerobic photometabolism of aromatic compounds by *Rhodopseudomonas* sp. Biochem. Soc. Trans. **4:**490–491.
27. **Ziegler, K., R. Buder, J. Winter, and G. Fuchs.** 1989. Activation of aromatic acids and aerobic 2-aminobenzoate metabolism in a denitrifying *Pseudomonas* strain. Arch. Microbiol. **151:**171–176.